Imipenem-resistance in *Serratia marcescens* is mediated by plasmid expression of KPC-2

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Abstract. – OBJECTIVE: Imipenem is a broad-spectrum carbapenem antibiotic with applications against severe bacterial infections. Here, we describe the identification of imipenem-resistant *Serratia marcescens* in our hospital and the role of plasmid-mediated KPC-2 expression in imipenem resistance.

MATERIALS AND METHODS: We used the modified Hodge test to detect carbapenemase produced in imipenem-resistant strains.

RESULTS: His resistance can be transferred to *E. coli* in co-culture tests, which implicates the plasmid in imipenem resistance. PCR amplification from the plasmid identified two products consistent with KPC-2 of 583 and 1050 bp that were also present in *E. coli* after co-culture. The restriction pattern for both plasmids was identical, supporting the transfer from the S. marcescens isolate to E. coli. Finally, gene sequencing confirmed KPC-2 in the plasmid.

CONCLUSIONS: Due to the presence of KPC-2 in the imipenem-resistant *S. marcescens*, we propose that KPC-2 mediates antibiotic resistance in the S. marcescens isolate.

Key Words:
Imipenem, Drug resistance, Serratia marcescens, Plasmid, KPC-2.

Introduction

The isolation of bacteria resistant to carbapenem antibiotics brings new challenges to the clinic[1]. Current statistics indicates that *Escherichia coli*, *Klebsiella pneumoniae*, and other enterobacteria are the main community- and hospital-acquired pathogenic infections[2]. Drug sensitivity tests[3] in our hospital suggest that drug resistance rates of *K. pneumoniae* to imipenem, meropenem, and ertapenem are 1.5, 2, and 2.9%, respectively. Multiple drug resistance rate to extended spectrum β-lactamase (ESBLs) and Ampc enzyme are 45%. The drug-resistance mechanism is considered to be mediated mainly by the production of carbapenemases, enzymes that break down these antibiotics[4]. Here, we describe the identification of imipenem-resistant *Serratia marcescens* and analyze its drug-resistance mechanism, concluding that plasmid-dependent *Klebsiella pneumoniae* carbapenemase-2 (KPC-2) is the main mediator of imipenem resistance.

Materials and Methods

Source of Drug-Resistant Bacteria

We identified 32 multi-drug-resistant bacteria in the clinical departments of our center after culture and drug sensitivity tests. The bacterial identification by Vitek instrument (bioMerieux, Marcy, l'Etoile, France) identified 20 cases of *E. coli*, eight of *K. pneumoniae,* and four of *S. marcescens*. The average minimum inhibitory concentration (MIC) for *S. marcescens* resistance to imipenem was 46.5 + 7.2 g/ml. We used *E. coli* ATCC25922 as quality control bacteria for the drug sensitivity test.

Detection of Carbapenemase Produced by Modified Hodge test

E. coli ATCC25922 suspension was diluted to 0.5 McF with sterile saline (1:10 dilution) and inoculated into MH agar plates and dried for 5 min. Three strains were inoculated into plates containing 10 μg imipenem paper with a 1 μl inoculating loop. Then, at least 20 mm lines were drawn from the center. The plate was incubated at 35°C for 20 hr. *E. coli* rapid growth near the tested strains indicates the production of carbapenemase. We used as positive control *K. pneumoniae* strains that produce KPC-2 and as negative control *K. pneumoniae* ATCC700603.

Co-culture

E. coli EC600 (rifampicin, MIC> 1000 μg/ml) was inoculated into 5 ml Luria-Bertani broth with

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imipenem-resistant *S. marcescens*. The mixture was shaken overnight. Then, 200 µl of donor strain, 100 µl of recipient strain and 600 µl of fresh LB broth were mixed together in 1.5 ml centrifuge tubes, and incubated at 35˚C for 4 hr. A small volume, mixed and without the mix, was transferred on Mueller-Hinton agar plates (cefotaxime 2.0 µg/ml, rifampin 512 µg/ml), and incubated at 35˚C for 24 hr. Single colonies were picked and incubated at 35˚C for 4 hr. VITEK bacterial identification was used for the biochemical identification of trans-conjugants and drug sensitivity test was carried out to detect the MIC of mixed and non-mixed *E. coli*.

Plasmid purification

Plasmid Extraction Kit (Axygen, Union City, CA, USA) was used according to the instructions manual. Isoelectric focusing electrophoresis: we used a polyacrylamide gel pH 3.5-9.5 (Pharmacia co., Kalamazoo, MI, USA) in a PhastSystem electrophoresis instrument and isoelectric focusing electrophoresis, DYY-II electrophoresis instrument (Beijing Six One Instrument Factory). The ultrasonic crushing method was used for plasmid extraction, and Nitrocefin (Oxoid, UK) was used as a substrate. In addition, conduction should follow reference[5]. The enzyme PI and known β-lactamase TEM-1 (5.4), SIIV-1 (7.6) and SIIV-5 (8.2) PI were compared as controls.

Plasmid Analysis: PCR and DNA Sequencing

BlaKPC and blaOXA-1 genes were amplified with specific primers (Table 1). The reaction volume was: 50 µl (final concentration was 1 x PCR buffer, Mg2+ 3.5 mmol/L, dNTPs 2 mmol/L, 500 mol/L primer, Taq 0.25U enzyme and 2 mu l template). For the PCR, we used PCR System GeneAmp 9600 (ABI co., Oyster Bay, NY, USA). The reaction parameters were 94˚C 5 min, 94˚C 45 sec. The annealing temperature was adjusted based on the primers, and could be extended to 72˚C 1 min, 30 cycles, and at last 72˚C 10 min. Amplified products were resolved by 1.2% gel electrophoresis, EB staining, and photographed under the gel imaging system. PCR products were purified according to Purification Test Kit Manual from Shanghai Shenergy gaming Biological Technology. Purified products were sent to Hangzhou biosune Biotechnology for sequencing. The sequencing results were compared in GenBank to identify the corresponding β-lactamase genes.

Analysis of plasmid DNA map: The plasmid DNA was extracted by alkaline lysis, and was carried out by the reference kit. *S. marcescens* and Escherichia coli plasmid DNA was dealt with a restriction endonuclease at 37˚C for 2 hr. Original plasmid and plasmid fragment dealt by restriction enzyme were added with a 0.8% agarose ethidium bromide gel, electrophoresed at 85 V constant voltage for 75 min, and observed under UV light.

Statistical Analysis

SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and data was expressed as mean ± standard deviation. The comparison between groups was made by *t*-test. $p<0.05$ was considered to be statistically significant.

Result

Results of the Improved Hodge Test

The Hodge test allows identifying bacterial strains that produce carbapenemases because they permit the growth of carbapenem-sensitive strains towards the antibiotic disc in the center of the plate (Figure 1). Imipenem creates an inhibition zone around the central disc that prevents the growth of *E. coli* ATCC25922. We identified four cases of *S. marcescens* that allow the growth of

Table I. PCR primer sequences.

Gene	Primers	Sequence(5′-3′)	Size(bp)
blaKPC	KPC-A KPC-B	TCTAAGTTACCGCGCTGAGG CCAGACGACCCCATACTCAT	583
bla_{KPC}	KPC-F KPC-R	GCTACACCTAGCTCCACCTTC TCAGTGCTCTACAGAAAACC	1050
bla_{OXA-1}	OXA-1-S OXA-1-AS	ACCCCTTAAAATTAAGCCC CTTGATTGAAGGGTTGGGCG	908

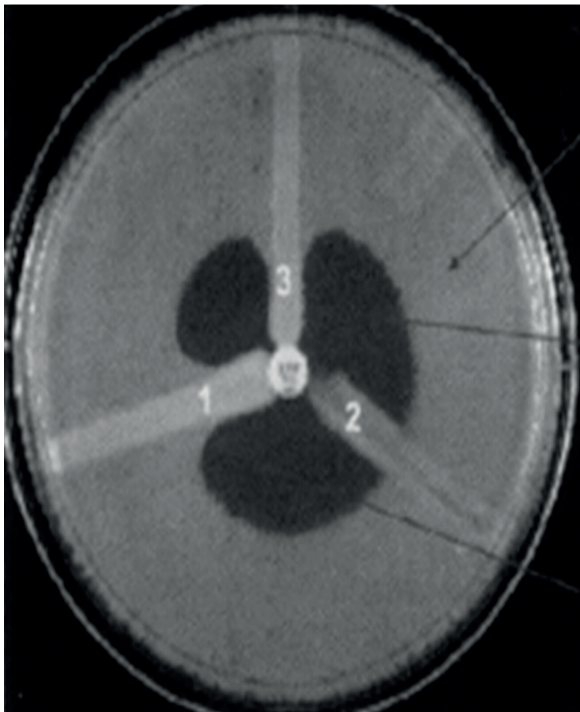

Figure 1. Improved Hodge test to detect carbapenem. 1, *Zuckerberg pneumonia* strains producing KPC-2 type. 2, *Z. pneumonia* strain ATCC700603. 3, Experimental strain to be identified).

E. coli ATCC25922, creating a cloverleaf indentation in the inhibition area. The positive Hodge test indicates that the *S. marcescens* isolates can produce carbapenemase.

Co-culture Experiment

The co-culture of the *S. marcescens* isolates and imipenem-sensitive *E. coli* EC600 produced similar resistance spectrum for *S. marcescens* (Table II). But the imipenem resistance rate and MIC for *E. coli* EC600 increased markedly (Table II). These results further support the production of carbapenemase by the *S. marcescens* isolates that result in significant benefits for *E. coli* EC600.

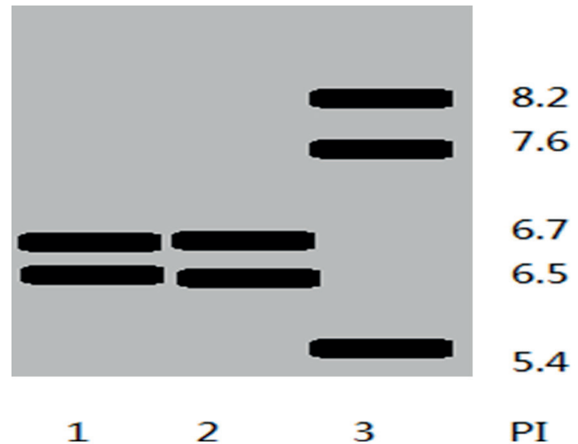

Figure 2. Joint Experiment and isoelectric focusing electrophoresis. 1, *S. marcescens*. 2, Joint carbapenem. 3, Known βphenolic amine TEM-1.

PCR and DNA Sequencing

Following co-culture and isoelectric focusing electrophoresis, we found a common pattern between *S. marcescens* and *E. coli*, with two bands running at 6.5 and 6.7 (Figure 2). PCR amplification from the purified plasmid produced two fragments of 583 and 1050 bp. The products isolated from *E. coli* after co-culture were the same as those purified from *S. marcescens*, but without blaOXA-1 and other fragments (Figure 3). Plasmid analysis showed that the plasmids isolated from *S. marcescens* and *E. coli* after co-culture had the same size, about 60 Kb and showed the same EcoRI restriction map. Gene sequencing of the PCR products confirmed the presence of the *KPC-2* gene (SEQ ID NO AY034847) in both *S. marcescens* isolates and *E. coli* after co-culture.

Discussion

So far, 11 isoforms of KPC enzymes have been identified, KPC-1 to 11[6]. Despite this diversity, the

Table II. Results of the co-culture.

	Drug rate to imipenem (%)		MIC (μg/ml)	
	before	after	before	after
S. marcescens	92.4±5.5	90.5±6.2	46.5±7.2	45.2±6.9
E. coli EC600	3.7±0.5	86.6±7.3#	1.7±0.6	42.3±5.8#
t	42.615	0.439	38.652	0.538
p	<0.001	0.626	<0.001	0.714

Notes: #comparison of E. coli EC600 engagement, $p<0.05$.

Figure 3. DNA blaKPC gene amplification. 1-4, *S. Marcescens*. 5-8, Joint carbapenem.

enterobacteriaceae of clinical relevance in China mainly produce KPC-2[7]. Rasheed and other American scholars isolated *Citrobacter freundii* and *Klebsiella oxytoca* from two patients that also produce the same KPC-2 isoform[8]. Sequencing the flanking sequence around *KPC-2* from *K. pneumoniae* isolated by Naas and cols[9], confirmed that it is the exact same gene as in *KPC-2* from American *Salmonella enterica*. The presence of the exact same *KPC-2* gene in the plasmids from two different bacteria is due to plasmid transfer, a phenomenon known as bacterial conjugation. *KPC-2* is almost identical to KPC-1 except for a Ser to Gly substitution at position 174 (S174G)[10]. The imipenem resistance of *S. marcescens* is mainly due to the production of β-lactamases (KPCs), which can hydrolyze the carbapenem antibiotics IMP-1, Imp-6, VIM-2, sme-1, and SME-2. This resistance is also associated with reduced levels of penicillin binding protein (PBP) combined with the loss or decreased of porin, which are critical for the transport of antibiotics[11].

The modified Hodge test is recommended by CLSI to detect the carbapenemase in enterobacteriaceae. The test sensitivity for ertapenem is higher than for meropenem and imipenem. The false positive results may be due to the lack of the ESBL outer membrane protein or the too large inocula[11]. Our conclusion is that all four cases of clay *Serratia* bacteria isolates produce carbapenemase. Conjugation experiments conferred *E. coli* EC600 the same drug resistance spectrum of *S. marcescens*. After the co-culture, we found the two same bands from *S. marcescens* and *E. coli*, which is different from previous results[13]. PCR amplification also isolated the same products from *S. marcescens* isolates and *E. coli* co-cultures, and none produced blaOXA-1 and other fragments similar to previous reports[14]. Gene sequencing confirmed the presence of *KPC-2* in *S. marcescens* isolates and *E. coli* co-cultures, supporting the role of *KPC-2* in the imipenem resistance.

At present, the infections caused by KPC-positive strains are rare and the number of effective antibacterial drugs is very limited. Although the drug sensitivity results show resistance to carbapenem antibiotics and other kinds of antibiotics, such as quinolones and aminoglycosides, the effect of the replacement antibiotic is less than ideal[15]. The research and development of β-lactamase inhibitors bring hope for treating strains that produce KPC. NXL104, LK-157 and BLI-489 are still under development, but preliminary results show that NXL104 can recover the antibacterial activity of various β-lactam antibiotics to strains producing KPC[16]. Also, the tricyclic carbapenem LK-157 has inhibitory activity for β-lactamase in class A and C[17]. BLI-489 is a bicyclic carbapenem molecule with inhibitory activity over various kinds of β-lactamase[18].

Conclusions

We suggest that the mechanism mediating *S. marcescens* resistance to imipenem requires plasmid-mediated KPC-2 expression.

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Conflict of interest

The authors declare no conflicts of interest.

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